Ornithinibacillus halophilus sp. nov., a moderately halophilic, Gram-stain-positive, endospore-forming bacterium from a hypersaline lake

Maryam Bagheri,1,2 Mohammad Ali Amoozegar,1,2 Peter Schumann,3 Maryam Didari,1 Malihe Mehrshad,1 Cathrin Spröer,3 Cristina Sánchez-Porro4 and Antonio Ventosa4

Correspondence
Mohammad Ali Amoozegar
amoozegar@ibrc.ir or
amoozegar@khayam.ut.ac.ir

1Extremophiles Laboratory, Department of Microbiology, Faculty of Biology and Center of Excellence in Phylogeny of Living Organisms, College of Science, University of Tehran, Tehran, Iran
2Microorganisms Bank, Iranian Bioresource Centre (IBRC), ACECR, Tehran, Iran
3DSMZ – German Collection of Microorganisms and Cell Cultures, Inhoffenstraße 7B, 38124 Braunschweig, Germany
4Department of Microbiology and Parasitology, Faculty of Pharmacy, University of Sevilla, 41012 Sevilla, Spain

A novel Gram-stain-positive, moderately halophilic bacterium, designated strain G8B\(^T\), was isolated from water of the hypersaline lake Aran-Bidgol in Iran and characterized taxonomically using a polyphasic approach. Cells of strain G8B\(^T\) were rod-shaped, motile and produced oval endospores at a terminal position in swollen sporangia. Strain G8B\(^T\) was strictly aerobic, catalase-positive and oxidase-negative. The strain was able to grow at NaCl concentrations of 0.5–12.5 % (w/v), with optimum growth occurring at 5–7.5 % (w/v) NaCl. The optimum temperature and pH for growth were 35–40 °C and pH 7.5–8.0, respectively. On the basis of 16S rRNA gene sequence analysis, strain G8B\(^T\) was shown to belong to the genus Ornithinibacillus within the phylum Firmicutes and showed closest phylogenetic similarity with Ornithinibacillus bavariensis WSBC 24001\(^T\) (97.6 %). The DNA G+C content of strain G8B\(^T\) was 36.9 mol%. The major cellular fatty acids of strain G8B\(^T\) were anteiso-C\(_{15}\):0, anteiso-C\(_{17}\):0, iso-C\(_{15}\):0 and iso-C\(_{16}\):0, and its polar lipid pattern consisted of phosphatidylglycerol, diphosphatidylglycerol, four unknown phospholipids and an unknown aminolipid. The isoprenoid quinones were MK-7 (98 %) and MK-8 (2 %). Strain G8B\(^T\) contained a peptidoglycan of type A4\(\beta\), L-Orn–D-Asp. All these features confirmed the placement of isolate G8B\(^T\) within the genus Ornithinibacillus. DNA–DNA hybridization experiments revealed a low level of relatedness (6 %) between strain G8B\(^T\) and Ornithinibacillus bavariensis DSM 15681\(^T\). On the basis of evidence from this study, a novel species of the genus Ornithinibacillus, Ornithinibacillus halophilus sp. nov., is proposed, with strain G8B\(^T\) (=IBRC-M 10683\(^T\)=KCTC 13822\(^T\)) as the type strain.

The genus Ornithinibacillus was proposed by Mayr \textit{et al.} (2006) to accommodate Gram-positive, strictly halotolerant to moderately halophilic and endospore-forming rod-shaped organisms with cell-wall peptidoglycan type A4\(\beta\), L-Orn–D-Asp. The major fatty acids are iso-C\(_{15}\):0 and anteiso-C\(_{15}\):0, the predominant menaquinone is MK-7 and the G+C content of its DNA is 36–41 mol%. At the time of writing, this genus comprises three species with validly published names: Ornithinibacillus bavariensis (type species) and Ornithinibacillus californiensis, isolated from pasteurized milk and coastal surface sediments, respectively (Mayr \textit{et al.}, 2006), and Ornithinibacillus contaminans, isolated as a contaminant of a human blood sample (Kämpfer \textit{et al.}, 2010).

The aim of the present study was to determine the taxonomic position of strain G8B\(^T\), isolated from a hypersaline lake in Iran, using a polyphasic approach. The resultant data indicated that this isolate represents a novel species of the genus Ornithinibacillus.

Strain G8B\(^T\) was isolated from the water of the hypersaline lake Aran-Bidgol (34° 18′–34° 45′ N, 51° 33′–52° 10′ E),
which is located near Kashan city in the centre of Iran, with an areal extent of about 650 km² in the dry season. The pH of the water of the lake is neutral (about pH 7.0–7.6) and its salinity reaches saturation. The main salts are NaCl, KCl, MgSO₄, MgCl₂ and Na₂SO₄. At the time of sampling, the temperature of the water was 32 °C and the pH 7.6. The strain was isolated by diluting the water sample in sterile 10 % (w/v) salts solution, plating on 7.5 % HM medium and incubating at 35 °C aerobically. The 7.5 % HM medium contained (g l⁻¹): NaCl, 60.7; MgCl₂, 6H₂O, 5.2; MgSO₄.7H₂O, 7.2; CaCl₂.2H₂O, 0.27; KCl, 1.5; NaHCO₃, 0.045; NaBr, 0.0195; proteose-peptone no. 3, 5; yeast extract, 10; and glucose, 1 (Ventosa et al., 1982). The pH of this medium was adjusted to 7.5. The strain was subsequently purified three times by plating on the same medium and maintained at −80 °C in 7.5 % HM medium without agar and supplemented with 30 % (v/v) glycerol. For phenotypic characterization and comparative purposes, reference strains Ornithinibacillus bavariensis DSM 15681T and Ornithinibacillus californiensis DSM 16628T were obtained from the DSMZ culture collection.

In order to characterize strain G8BT the recommended minimal standards for describing new taxa of aerobic, endospore-forming bacteria were followed (Logan et al., 2009).

Cell morphology was examined using an Olympus BX41 microscope equipped with phase-contrast optics. Gram staining was performed by the Burke method (Murray et al., 1994) and the result was confirmed by the KOH test (Baron & Finegold, 1990). The presence of endospores was investigated by using the Schaeffer–Fulton staining method (Murray et al., 1994). Motility was analysed by the wetmount method (Murray et al., 1994). Catalase, oxidase and urease activities, nitrate reduction, hydrolysis of aesculin, production of indole, and methyl red and Voges–Proskauer tests were carried out according to Smibert & Krieg (1994). Hydrolysis of Tweens 40, 60 and 80 was examined as described by Harrigan & McCance (1976). Determination of acid production from carbohydrates, as well as utilization of carbon and energy sources, was performed as recommended by Ventosa et al. (1982). Antimicrobial susceptibility tests were performed on Mueller–Hinton agar plus 7.5 % (w/v) sea salts (Ventosa et al., 1982) seeded with a bacterial suspension containing 1.5 × 10⁸ c.f.u. ml⁻¹ using discs (HiMedia) impregnated with various antimicrobial compounds. The plates were incubated at 35 °C for 48 h and the inhibition zone was interpreted according to the manufacturer’s manual. To determine the optimal temperature and pH for growth of the strain, nutrient broth was incubated at 15–60 °C at intervals of 5 °C and at pH 6–9 at intervals of 0.5 pH units. pH was adjusted using Tris/HCl buffer. Growth at different NaCl concentrations (0, 0.5, 1, 2.5, 5, 7.5, 10, 12.5 and 15 %, w/v) was tested on HM medium at pH 7.5. Growth was monitored by turbidity at OD₆₀₀ using a spectroscopic method (model UV-160 A; Shimadzu). Other physiological and biochemical tests were performed as described previously (Mata et al., 2002; Quesada et al., 1984; Ventosa et al., 1982).

Strain G8BT was Gram-stain-positive, motile and strictly aerobic. Oval endospores were produced at the terminal position in swollen sporangia. Cells were rods with a width of 0.5–0.6 μm and length of 4.0–20.0 μm. When grown for 2 days at 35 °C on 7.5 % HM medium, colonies were circular, convex, entire, smooth, creamy white and with a diameter of 0.6 mm. This isolate was moderately halophilic, growing in media containing 0.5–12.5 % (w/v) NaCl and optimally in media containing 5.0–7.5 % (w/v) NaCl. Strain G8BT grew between pH 6.5 and 8.5 and optimally in media with pH 7.5–8.0. Other phenotypic features are included in Table 1 and the new species description.

Genomic DNA from strain G8BT was isolated using the method described by Marmur (1961). The 16S rRNA gene was amplified by PCR with the forward primer F27 and the reverse primer R1488. Direct sequence determination of the PCR-amplified DNA was carried out using an automated DNA sequencer model ABI 3130XL (Applied Biosystems) by the SeqLab Laboratory (Göttingen, Germany).

16S rRNA gene sequence analysis was performed with the ARB software package (Ludwig et al., 2004). The 16S rRNA gene sequence was aligned with the published sequences of closely related bacteria and the alignment was confirmed and checked against both primary and secondary structures of the 16S rRNA molecule using the alignment tool of the ARB software package. Phylogenetic trees were constructed using three different methods: maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981), algorithms integrated in the ARB software for phylogenetic inference. The 16S rRNA gene sequences used for phylogenetic comparisons were obtained from the GenBank database and their strain designations and accession numbers are shown in Fig. 1.

The almost-complete 16S rRNA gene sequence (1487 bp) of strain G8BT was obtained and used for initial blast searches in GenBank and phylogenetic analysis. The identification of phylogenetic neighbours and calculation of pairwise 16S rRNA gene sequence similarity were achieved using the EzTaxon-e server (http://eztaxon-e.ezbiocloud.net/; Kim et al., 2012). The phylogenetic tree based on the maximum-parsimony algorithm revealed that strain G8BT was included in a cluster constituted by species of the genus Ornithinibacillus (Fig. 1). The closest relative of strain G8BT was Ornithinibacillus bavariensis WSBC 24001T, with a 16S rRNA gene sequence similarity of 97.6 %. 16S rRNA gene sequence similarities of strain G8BT to Ornithinibacillus californiensis MB-9T and Oceanobacillus profundus CL-MP28T were 97.0 and 96.8 %, respectively. The level of 16S rRNA gene sequence similarity between strain G8BT and other Gram-positive endospore-forming bacilli was less than 96 %. Maximum-likelihood and neighbour-joining methods resulted in highly similar tree topologies (Fig. 1) and
confirmed the phylogenetic cluster formed by strain G8B<sup>T</sup> and the type strains of species of the genus *Ornithinibacillus*.

For determination of DNA base composition and DNA–DNA hybridization, DNA was isolated using a French pressure cell (Thermo Spectronic) and was purified by chromatography on hydroxyapatite as described by Cashion et al. (1977). DNA–DNA hybridization was carried out as described by De Ley et al. (1970) incorporating the modifications described by Huss et al. (1983) using a Cary 100 Bio UV/VIS spectrophotometer.
equipped with a Peltier-thermostatted 6 × 6 multicell changer and a temperature controller with an in situ temperature probe (Varian).

The level of DNA–DNA relatedness between strain G8B$^\text{T}$ and *Ornithinibacillus bavariensis* DSM 15681$^\text{T}$ was 6.0 %. According to Wayne *et al.* (1987), less than 70 % DNA–DNA relatedness is considered to be the threshold value for the delineation of genospecies, so the value obtained is low enough to differentiate strain G8B$^\text{T}$ from its closest relative, *Ornithinibacillus bavariensis* DSM 15681$^\text{T}$.

The DNA G + C content was determined by reversed-phase HPLC of nucleosides according to Mesbah *et al.* (1989). The G + C content of the DNA of strain G8B$^\text{T}$ was 36.9 mol%. This value is within the range described for the genus *Ornithinibacillus* (36.0–41.0 mol%) (Mayr *et al.*, 2006).

Cell biomass for analysis of the cell-wall peptidoglycan, isoprenoid quinones and polar lipids was obtained by cultivation on 7.5 % HM agar at 35 °C. Preparation and hydrolysis of the cell wall was carried out using the method of Schleifer (1985) and the interpeptide bridge in the cell-wall peptidoglycan was analysed by using the method described by Schleifer & Kandler (1972). Cell-wall hydrolysates were separated by one- or two-dimensional chromatography on cellulose thin-layer plates (Merck). The quantitative ratio of amino acids in the peptidoglycan hydrolysate (4 M HCl, 100 °C, 16 h) was determined by GC as described by MacKenzie (1987). Isoprenoid quinone analysis was carried out as described by Monciardini *et al.* (2003). Polar lipids were analysed as described by Groth *et al.* (1996).

The cell-wall peptidoglycan was of type A4β, L-Orn–D-Asp, in accordance with that reported for the genus *Ornithinibacillus* (Mayr *et al.*, 2006). The major isoprenoid quinone of strain G8B$^\text{T}$ was MK-7 (98 %), while MK-8 (2 %) was also present in minor amounts. The respiratory lipoquinones of strain G8B$^\text{T}$ were typical of those found in members of the genus *Ornithinibacillus* (Mayr *et al.*, 2006; Kämpfer *et al.*, 2010).

The polar lipids detected as major compounds were phosphatidylglycerol, diphosphatidylglycerol, four unknown phospholipids and an unknown aminolipid (Fig. S1, available in IJSEM Online). The polar lipid profile of strain G8B$^\text{T}$ is more similar to that of *Ornithinibacillus contaminans* than to that of *Ornithinibacillus bavariensis*. Similarly to *Ornithinibacillus contaminans* aminophospholipids could not be detected in strain G8B$^\text{T}$; however, unknown glycolipids and unknown lipids that were reported for *Ornithinibacillus bavariensis* and *Ornithinibacillus contaminans* (Mayr *et al.*, 2006; Kämpfer *et al.*, 2010) were absent in strain G8B$^\text{T}$.

The whole-cell fatty acid composition was determined by GC using the Microbial Identification System (MIDI, Version 6.1; Identification Library TSBA40 4.1; Microbial ID). Cells were cultured on 5.0 % HM medium at 35 °C for 10 days, subcultured on 7.5 % HM agar at 35 °C and cultivated on 7.5 % HM agar at 35 °C. Preparation and hydrolysate (4 M HCl, 100 °C, 16 h) was determined by GC as described by Groth *et al.* (1996). The quantitative ratio of amino acids in the peptidoglycan hydrolysate (4 M HCl, 100 °C, 16 h) was determined by GC as described by MacKenzie (1987). Isoprenoid quinone analysis was carried out as described by Monciardini *et al.* (2003). Polar lipids were analysed as described by Groth *et al.* (1996).

The level of DNA–DNA relatedness between strain G8B$^\text{T}$ and *Ornithinibacillus bavariensis* DSM 15681$^\text{T}$ was 6.0 %. According to Wayne *et al.* (1987), less than 70 % DNA–DNA relatedness is considered to be the threshold value for the delineation of genospecies, so the value obtained is low enough to differentiate strain G8B$^\text{T}$ from its closest relative, *Ornithinibacillus bavariensis* DSM 15681$^\text{T}$.

The DNA G + C content was determined by reversed-phase HPLC of nucleosides according to Mesbah *et al.* (1989). The G + C content of the DNA of strain G8B$^\text{T}$ was 36.9 mol%. This value is within the range described for the genus *Ornithinibacillus* (36.0–41.0 mol%) (Mayr *et al.*, 2006).

**Description of *Ornithinibacillus halophilus* sp. nov.**

*Ornithinibacillus halophilus* (ha.lo’phi.lus. Gr. n. hals, halos salt; Gr. adj. philos loving; N.L. masc. adj. halophilus salt-loving).

Cells are Gram-stain-positive, motile, endospore-forming rods, 0.5–0.6 × 4.0–20.0 µm in size. Oval endospores are produced at a terminal position in swollen sporangia. Colonies are circular, convex, entire, smooth, creamy white and 0.6 mm in diameter on 7.5 % HM agar medium after 48 h at 35 °C. Strictly aerobic. Moderately halophilic, growing over a wide range of NaCl concentrations (0.5 to 12.5 %, w/v, NaCl), with optimal growth at 5–7.5 % (w/v) NaCl. Grows at 20–55 °C (optimum, 35–40 °C) and pH 6.5–8.5 (optimum, pH 7.5–8.0). Catalase-positive and oxidase-negative. Starch is hydrolysed but casein, gelatin, DNA, and Tweens 40, 60 and 80 are not. Nitrate is not reduced. Indole and H$_2$S are not produced. Acid is produced from β-fructose, α-glucose, lactose, ribose and glycero, but not from galactose, sucrose, maltose or α-mannitol. Methyl red, Voges–Proskauer, urease, β-galactosidase, lysine decarboxylase, ornithine decarboxylase, arginine dihydrolase and phenylalanine deaminase tests are negative. The following compounds are utilized as sole source of carbon and energy: raffinose, starch and alanine. The following compounds are not utilized as sole source of carbon and energy: arabinose, α-glucose, α-ribose, sucrose,
mannotol, galactose, D-mannose, melibiose, D-fructose, cellobiose, trehalose, glycerol, L-asparagine, L-arginine, L-histidine, L-leucine, glycine, methionine, phenylalanine, proline, cysteine, tyrosine and valine. Sensitive to amoxi-
cillin (30 μg), ampicillin (30 μg), bacitracin (10 μ), carbenicillin (100 μg), gentamicin (30 μg), nitrofurantoin (300 μg), tetracycline (30 μg), tobramycin (10 μg) and rifampicin (5 μg). Resistant to amikacin (30 μg), neomycin (30 μg), kanamycin (30 μg), nalidixic acid (30 μg) and polymyxin B (100 U). The major isoprenoid quinone is MK-7. The peptidoglycan is of type A4β, L-Orn–D-Asp. Major cellular fatty acids are anteiso-C₁₅ : ₀, anteiso-C₁₇ : ₀, iso-C₁₅ : ₀ and iso-C₁₆ : ₀. Polar lipids are diphasphatidyl-
N-glycerol, phosphatidylglycerol, four unknown phospholi-
pids and an unknown aminolipid.

The type strain, G8β³T (=IBRC-M 10683T = KCTC 13822T), was isolated from Aran-Bigdel hypersaline lake in Iran. The DNA G+C content of the type strain is 36.9 mol% (HPLC).

Acknowledgements

This work was supported by grants from Iranian Biological Resource Centre (IBRC) (MI-1388-04) (to M. A. A.), from the Spanish Ministerio de Economía y Competitividad (CGL2010-19303) that includes European Funds (FEDER), the National Science Foundation (Grant DEB-0919290) and Junta de Andalucía (P10-CVI-6226) (to A. V.). We thank Bettina Straußler for excellent technical assistance.

References